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PRINCIPAL INVESTIGATOR: Dr. David E. Wemmer

CONTRACTING ORGANIZATION: Regents of the University of California
Berkeley CA 94720

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Dr. David E. Wemmer

E-Mail: dewemmer@LBL.gov

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14. ABSTRACT
The mouse pron protein peptide (residues 89-143 with the substitution of Leu for Pro atresidue 101) induces prion disease in sensitized mice. Samples of this peptide, isotopelabeled with 15N, were prepared by expression of a fusion in E.coli, cleaved to yield anunmodified peptide, and then fibrillized. Hydrogen exchange was allowed to occur in thefibrils for periods from 1 hour to 6 weeks, repeating initial experiments to verify theexchange behavior. The extent of exchange was monitored using peak intensities in 15N-1H HSQCnuclear magnetic resonance spectra in DMSO/D2O/TFA solutions. The results are consistent withprevious measurements that we did. Analogous experiments were also done with unlabeledpeptide in fibrils with the analysis done by mass spectroscopy. Fragmentation was improvedrelative to previous experiments by using a combination of two proteases. With the improvedcoverage it was possible to show that the same protected regions as determined by NMR couldbe identified.

15. SUBJECT TERMS
prion protein peptide, solid state NMR, hydrogen exchange, mass spectroscopy

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INTRODUCTION

The central effort in this project has been the elucidation of the conformation of the prion protein fragment containing residues 89-143 in fibrils (previously demonstrated to induce prion disease upon injection into mice, Kaneko et al., 2000). By probing the exposure of amides using hydrogen exchange, detected both with NMR and mass spectroscopy we are enabling a comparison of the *in vitro* generated peptide and protein models with real prions produced *in vivo*. Of particular interest is to determine the relationship between core structure features and strain behavior. Understanding the fundamental steps of initiation and propagation of the disease associated conformation may lead to new methods for preventing conversion to the pathogenic form. The experimental work has continued to confirm and extend hydrogen exchange of backbone amides, detected both with NMR and mass spectroscopy. Samples are mouse prion protein residues 89-143 containing a P to L substitution at residue 101 (a mutation that is associated with disease). This remains the simplest bioactive model for prion disease. Samples were prepared both through chemical synthesis of the 55 residue peptide using standard solid-phase methods, and through expression of a fusion peptide in *E.coli* cells that is then purified and cleaved to give the native 55 residue peptide.

BODY

Production of PrP(89-143)P101L (=P_{55}) samples

The protocols we have applied for preparing samples in *E.coli* have continued to work well. For the work during this extension period, we have done expression with ^{15}N ammonia to obtain uniformly ^{15}N labeled samples for NMR detected hydrogen exchange. Unlabeled fibrils were made and handled in the same way for mass spectroscopy detected hydrogen exchange. Additional unlabeled peptide was also sent to another lab (Stubbs at Vanderbilt) that has been making magnetically ordered fibrils for diffraction work, but which would also be useful for solid state NMR.

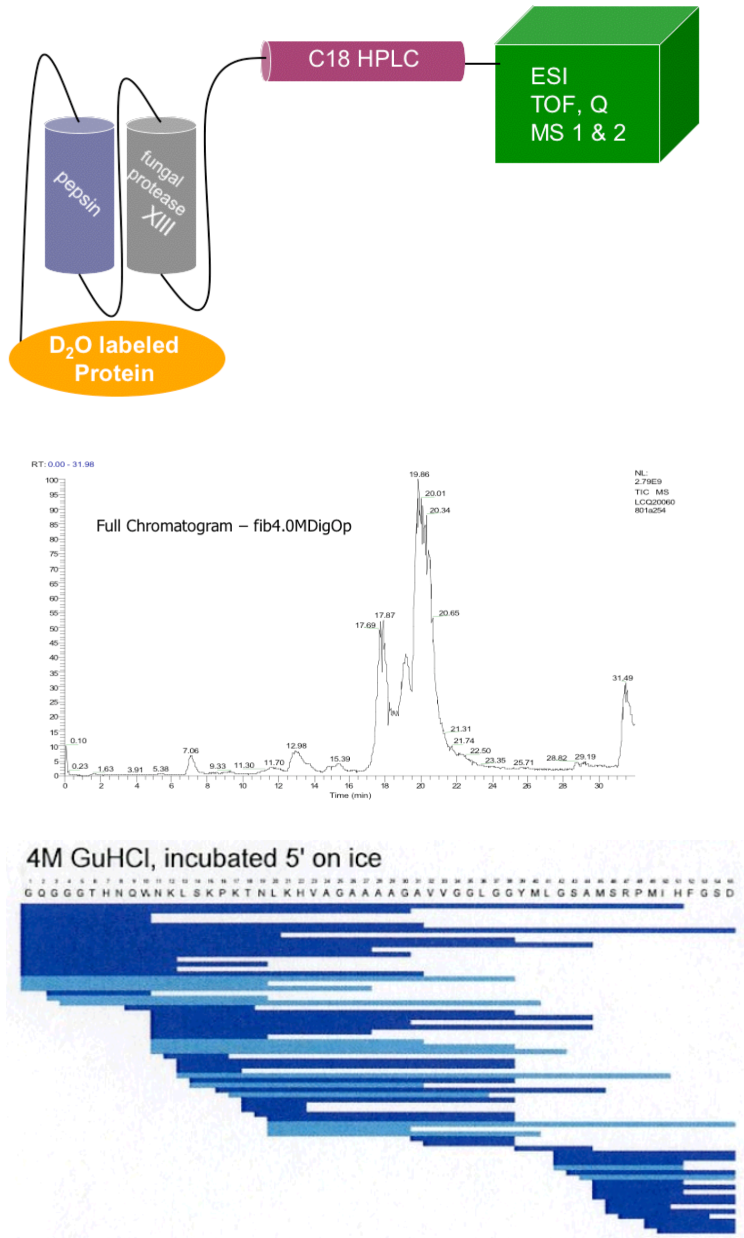
NMR based quenched hydrogen exchange of PrP 89-143 P101L

To make sure that the NMR detected hydrogen exchange was giving reliable and reproducible results, we repeated the exchange measurements on ^{15}N labeled fibrils using the same approach described in previous reports. Briefly protonated fibrils were prepared by aggregation in phosphate buffered saline with acetonitrile in the cold for 3 weeks, then were collected, resuspended in pD 7.5 10 mM phosphate buffer and incubated at room temperature. Fibrils were pelleted, washed with cold D_2O, then lyophilized. The dry fibrils were then dissolved in DMSO with 5% D_2O, 0.03% TFA-D, and analyzed by NMR. Assignments were described in previous reports. The results of these experiments were very similar to those reported previously, verifying the slowly exchanging amides (those in hydrogen bonds in the fibrils), and the multiexponential decay seen for some residues. These experiments show that the approach is reproducible.

Mass spectroscopic hydrogen exchange of PrP 89-143 P101L

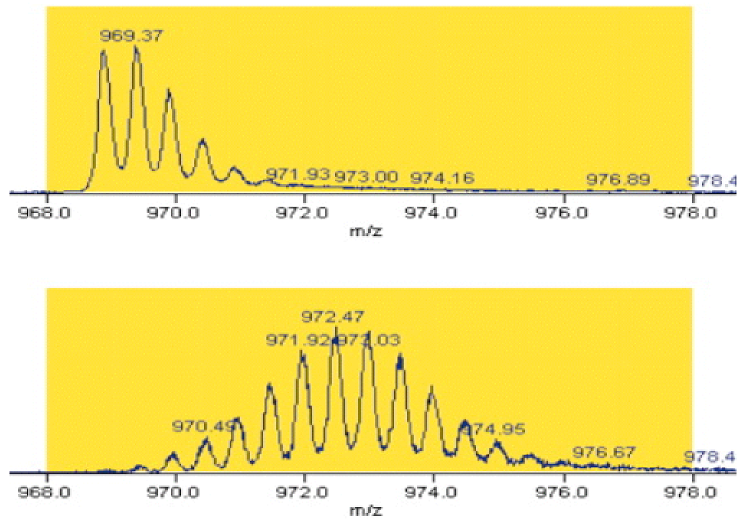
Samples of unlabeled fibrils were prepared in the same way that the ^{15}N labeled ones were, and then were subjected to the same exchange treatment. However in this case the samples were frozen, then taken to UCSD for analysis using an automated system in the laboratory of Prof. Virgil Woods (Englander et al., 2003). Previous experiments used

only a pepsin column for fragmenting the PrP peptide, but the number of fragments obtained was relatively low. To improve the fragmentation a second column, with fungal protease XIII was added in series to further fragment the initial pepsin peptides. Many different conditions were tested (different concentrations of denaturant, different denaturant and for increasing times of digestion). In fact many more peptides were generated. These were separated on the in-line C18 reverse phase HPLC column, and the eluant was run directly into the mass spectrometer to detect the mass change. Experiments were first done with non-deuterated peptide, and secondary fragmentation was done in the mass spectrometer to get a reliable identification of the peptides being observed. Although there is overlap in elution time, the separation of different peptides in the mass dimension reduces the overlap problems for the analysis. Once the peptides were identified, and the optimum digestions conditions were determined then the experiments were repeated using peptide that from fibrils that had been exposed to D_2O for exchange in, for variable amounts of time. These were then dissolved in denaturant, and subjected to the same fragmentation and mass measurements as for the unlabeled peptide. As expected, as deuterium exchanges in the peptides increase in their average mass. By comparing different peptides it is possible to localize the regions which have the most amides that are protected against exchange. The analysis of these data is consistent with the same regions as seen in the NMR detected experiments having the highest level of protection against exchange. The mass spectra were analyzed to quantify the extent of exchange observed. To do this a



Top: schematic of the system used for proteolytic fragmentation and mass spectroscopic hydrogen exchange determination. Middle: mass spec total ion chromatogram showing the profile of elution times for peptide fragments. Bottom: peptide map, bars indicating peptides that were observed. Dark bars indicate peaks that were clearly identifiable based on masses of fragments, lighter bars also identified but with less verification.

determination of the level of 'back exchange' during the process of fragmentation and separation needs to be determined. A measurement was therefore also done with fully deuterated peptide (not fibrilized). However the measurements suggested that the level of deuteration was less than expected, so this determination will have to be redone. Samples have been prepared for a repeat run of both the exchange in and the control for back exchange. These experiments will give a more accurate determination of the number of amides protected against exchange but should not change the determination of the residues that are protected against exchange. With the peptide fragmentation observed in these experiments the localization of protected amides should be possible to within 5-10 amino acids, depending on the specific region and level of fragmentation. Experiments with the full length prion protein should make possible a comparison of protected regions to determine whether the hydrogen bonding in the *in vivo* PrP aggregated material, on in other *in vitro* aggregated peptide models is the same as in this small model system. Comparison of different strains may also be informative.



Mass spectra of a peptide fragment from PrP 89-101, +2 charge state. Top: no deuteration shows the natural abundance isotope distribution; Bottom: after deuteration, for this peptide about 7 deuterons (on the average) have been exchanged in.

KEY RESEARCH ACCOMPLISHMENTS

•repeated a full set of hydrogen exchange measurements on P_{55} peptide in fibrils, using NMR verifying the reliability of the measurements.

•carried out a set of hydrogen exchange measurements using mass spectroscopy after improving the fragmentation. Reasonable localization was possible, and the data verified that protection against exchange occurred in the same regions as observed by NMR.

REPORTABLE OUTCOMES

A paper is being written on the results of the hydrogen exchange measurements, publication of the NMR results will wait until the complementary results from the mass spectroscopy based measurements are completed.

CONCLUSIONS

We have verified the reliability of the NMR based hydrogen exchange measurements on fibrils of PrP 89-143 P101L. We have now demonstrated that complementary measurements can be done with mass spectroscopy, with a sufficient level of fragmentation that regions protected against exchange can be identified. The first set of measurements are consistent with the results from NMR. The mass spectroscopy approach have good potential to be applied to longer PrP constructs, and in particular natural PrP forms that cannot be prepared in isotope labeled forms.

REFERENCES

Kaneko, K., Ball, H.L., Wille, H. Zhang, H., Groth, D., Torchia, M., Tremblay, P., Safar, J., Prusiner, DeArmond, S.B., Baldwin, M.A., Cohen, F.E. A Synthetic Peptide Initiates Gerstmann-Sträussler-Scheinker (GSS) Disease in Transgenic Mice. J. Molec. Biol. **295**, 997-1007 (2000).

Englander, J.J., Del Mar, C., Li, W., Englander, S.W., Kim, J.S., Stranz, D.D., Hamuro, Y., Woods, V.L. Jr. Protein structure change studied by hydrogen-deuterium exchange, functional labeling, and mass spectrometry. Proc. Natl. Acad. Sci. **100**, 7057-7062 (2003).